IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants: Yukoh HIEI et al

Serial No.: 10/089,695 Group: 1638

Filed: May 21,2002 Examiner: Helmer

For: METHOD FOR PRODUCING EFFICIENTY OF GENE TRANSFER INTO PLANT CELLS

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C., 20231

Sir:

I, Yukoh HIEI, a nation of Japan, residing at c/o Japan Tobacco Inc., Plant Innovation Center, 700, Higashibara, Iwata-shi, Shizuoka 438-0802, Japan, do hereby declare as follows:

I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Office Action dated April 25, 2007, in which claims 1, 3-7, 12, 14-15, 17-18, 20-21 and 23-24 are rejected.

To show the patentability of the present invention, I carried out the experiments described below.

Materials and Methods

(1) *Agrobacterium* Strain and Plasmid

As the *Agro*bacterium and its vector, LBA4404(pSB134) (Hiei and Komari, 2006) was used. The T-DNA region of pSB134 has a hygromycin-resistant gene (hpt) regulated by maize ubiquitin promoter and a GUS gene regulated by the 35S promoter of CaMV and having the first intron of the catalase gene of castor-oil plant.

(2) Sample Varieties and Tissue

As the sample varieties, Yukihikari and Koshihikari, which are the varieties of Japonica rice, were used. As the sample tissue, immature embryo was used. The preparation method of the tissue is the same as that described in the specification of the present patent application.

(3) Heat-centrifugation Treatment

Rice immature embryo was placed in a 1.5 ml centrifugal tube containing 1 ml of sterilized water. The tube was transferred to a thermostat water bath and heat-treated at 46°C for 5 minutes. After cooling on ice for about 5 minutes, the tube was subjected to centrifugation treatment for 10 minutes at 760 xg, 1,000 xg or 2,000 xg. In addition to these 3 test groups, 2 control groups, that is, a non-treated group and a group subjected to only the heat treatment (46°C for 5 minutes) were also included, thus totally 5 groups were tested. In each of the groups, 15 embryos were used. After the heat-centrifugation treatment, the immature embryos were infected with *Agrobacterium*.

(4) Infection of *Agrobacterium* and Co-culturing

The method of infection of the immature embryos with *Agrobacterium*, the method of co-culturing and the method of GUS assay of the immature embryos after the co-culturing were the same as described in specification of the present patent application. In the present test, the GUS expression levels in the immature embryos were expressed in values as GUS Activity Index as follows: Each of the immature embryos was then visually examined for the percentage of the sum of the blue areas to the total surface area of the scutellum. A score was given according to the percentage; score 0.0 was given for 0%, score 0.5 for between 0% and 1%, score 5.5 for between 1% and 10%, score 17.5 for between 10% and 25%, score 37.5 for between 25% and 50%, score 62.5 for between 50% and 75%, and score 87.5 for 75% and 100%. The average of the scores in an experimental plot was recorded as the GUS Activity Index. The co-culturing was carried out for 6 days.

Results and Discussion

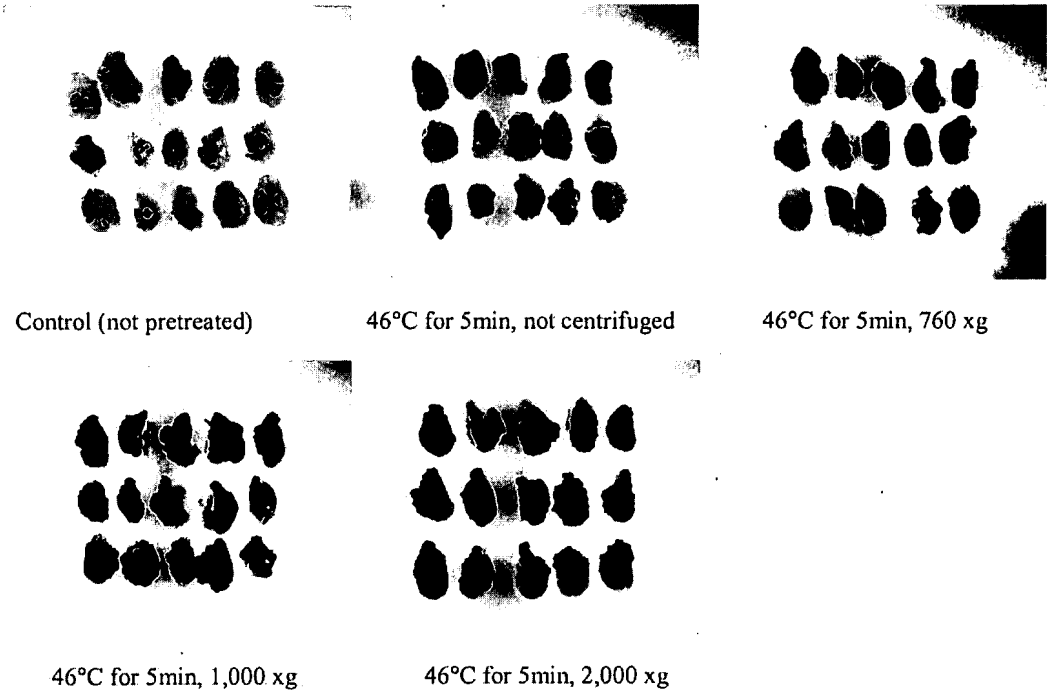
A tendency was observed that the growth of the hypocotyl is inhibited and the scutellum is grown during the co-culturing in the immature embryos subjected to centrifugation. This tendency was prominent in the groups subjected to the centrifugation at 1,000 xg and 2,000 xg, respectively. The state of GUS expression in the immature embryos after the co-culturing is shown in Figure 1 and 2. The percentage of the area in the scutellum, which showed GUS expression was increased by the heat treatment when compared with the non-treated group (Figures 1 and 2, and Table 1). In cases where the centrifugation at 760 xg was added to the heat treatment, increase in the GUS-expressed area was scarcely observed (Figure 1 and 2, and Table 1). In contrast, in the groups subjected to 1,000 xg and 2,000 xg, respectively, in addition to the heat treatment, the percentage of the GUS-expressed area was apparently increased when compared with the cases where only the heat treatment was performed (Figures 1 and 2, and Table 1). Thus, in cases where centrifugation treatment is added to the heat treatment, the centrifugation treatment at 1,000 xg or more has an effect to prominently increase the gene transfer efficiency.

Cited Reference

Hiei Y, Komari T (2006) Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*. Plant Cell, Tissue Organ Culture 85, 271-283

Table 1. Transient GUS activity in immature embryos after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with heat and/or centrifugation.

Pretreatment		GUS Activity Index	
		Variety	
Heat	Centrifugation	Yukihikari	Koshihikari
–	–	7.1	2.8
46°C for 5min	–	13.5	10.3
46°C for 5min	760 xg	14.3	11.6
46°C for 5min	1,000 xg	38.5	41.5
46°C for 5min	2,000 xg	55.8	75.8



Control (not pretreated) 46°C for 5min, not centrifuged 46°C for 5min, 760 xg

46°C for 5min, 1,000 xg 46°C for 5min, 2,000 xg

Figure 1. Histochemical GUS expression in immature embryos of Yukihikari after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with heat and/or centrifugation.

Control (not pretreated) 46°C for 5min, not centrifuged 46°C for 5min, 760 xg

46°C for 5min, 1,000 xg 46°C for 5min, 2,000 xg

Figure 2. Histochemical GUS expression in immature embryos of Koshihikari after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with heat and/or centrifugation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 24 day of October, 2007

Yukoh Hiei

Yukoh HIEI